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Interaction of Complex Pollutant Mixtures and Particulates in Causation of Pulmonary Disease

IIT Research Inst., Chicago, IL

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

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INTERACTION OF COMPLEX POLLUTANT MIXTURES AND PARTICULATES IN CAUSATION OF PULMONARY DISEASE

by

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Studies were conducted to determine the effects of inhalation exposure to particulate and gaseous air pollutants on the resistance to experimental bacterial pneumonia. Changes in resistance to this respiratory infection, expressed as changes in mortality rates and survival time, served as the most sensitive and consistent indicators of damage produced by the exposure. Using these parameters it was possible to rank the effects of single 3-hr inhalation exposure to the particulate pollutants. Among the particulate pollutants included in these studies cadmium sulfate and cadmium nitrate were most toxic followed in decreasing order of toxicity by copper, aluminum and magnesium sulfate or nitrate aerosols. The metallic cation appeared to be most important in altering the resistance to infection. Multiple 3-hr exposures to zinc or cupric sulfate similarily reduced the resistance at significantly lower concentrations of the pollutants.

Nitrogen dioxide and ozone mixtures reduced the resistance to bacterial pneumonia after 6-month inhalation exposure to various regimens of these photochemical oxidant pollutants.

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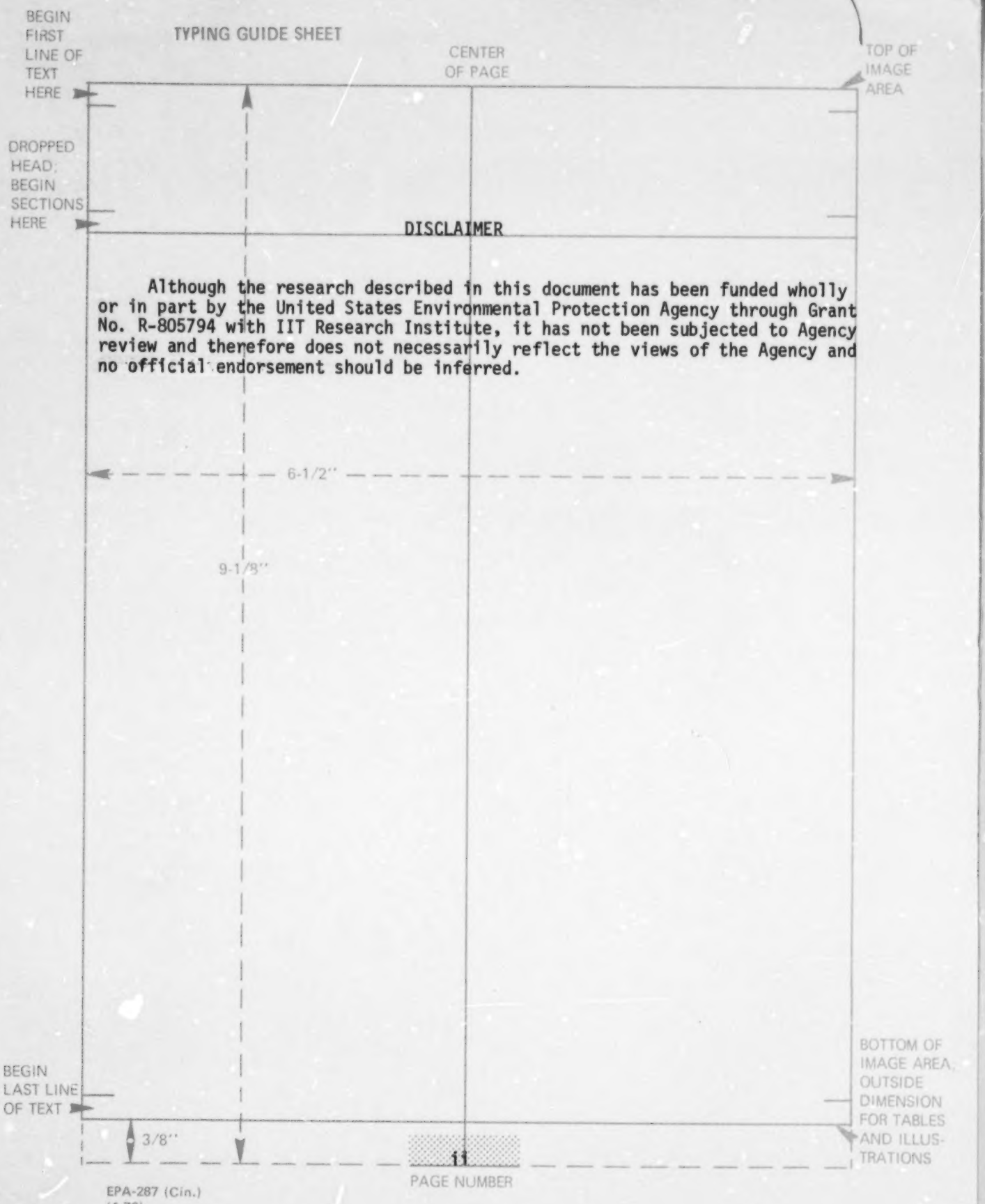
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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory conducts a coordinated environmental health research program in inhalation toxicology, genetic toxicology, neurotoxicology, developmental and experimental biology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, water pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides and other chemical pollutants. The Laboratory participates in and provides data for the development and revision of criteria documents on pollutants for which national ambient air quality and water quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

In this research, studies were conducted to determine the effects of inhalation exposure to particulate and gaseous air pollutants on the resistance to experimental bacterial pneumonia.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

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Nitrogen dioxide and ozone mixtures reduced the resistance to bacterial pneumonia after 6-month inhalation exposure to various regimens of these photochemical oxidant pollutants.

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SECTION 1

INTRODUCTION

Changes in the resistance to respiratory infection provide a highly sensitive experimental animal model system for studies of the effects of air pollutants at concentrations resembling those found in urban environment. The model reflects the impairment of the host defense mechanisms by the combined exposures to air pollutants and to infectious bacterial aerosol.

The objective of this project was to determine the effects of single or multiple short-term exposures to particulate aerosols representing various sulfates and nitrates on the resistance to streptococcal pneumonia and to study the changes in resistance to infection resulting from long term exposures to nitrogen dioxide and ozone mixtures. In these experiments it was of special interest to determine the effects of short-duration peaking of the mixture superimposed on a continuous exposure to low concentrations of nitrogen dioxide.

Results of these studies were summarized in a series of quarterly progress reports submitted at regular intervals to the Environmental Protection Agency. In addition phases of the studies were reported and published by the principal investigator.

Symposium on Experimental Models for Pulmonary Research, February 5-7, 1979, Hilton Head Island, SC. Report No. EPA-600/9-79-022.

International Conference on Critical Current Issues in Environmental Health Hazards, March 4-7, 1979, Tel-Aviv, Israel.

Interaction Between Environmental Pollutants and Respiratory Infections, R. Ehrlich, Environmental Health Perspectives 35, 89-100, 1980.

Changes in Susceptibility to Respiratory Infections Caused by Exposures to Photochemical Oxidant Pollutants. In: Proceedings International Symposium on Biomedical Effects of Ozone and Related Photochemical Oxidants, March 14-18, 1982, Pinehurst, N.C. Advances in Modern Environmental Toxicology (in press).

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SECTION 2

CONCLUSIONS

Results of the studies reconfirmed previous observations that increase in mortality rates resulting from bacteria pneumonia is reproducible and sensitive indicator of adverse effects of air pollutants.

A single 3-hr exposure to cadmium, cupric or aluminum sulfate or nitrate resulted in significant increase in mortalities and decrease in mean survival time in mice infected with streptococcus aerosol. These effects were seen at concentrations of approximately 0.40 mg/m^3 cadmium, 0.85 mg/m^3 cupric or 1.80 mg/m^3 aluminum sulfates or nitrates. In all studies the metallic cation appeared to be the fraction of the pollutant which was effective in altering the resistance to the infection. Several other sulfate, nitrate and nitrite pollutants used in the experiments had no effect on the mortality rates. Results of this phase of the program confirmed the utility of this animal model in ranking the toxic effects of inhalation of particulate pollutants.

A 6 month continuous (24 hr/day, 7 days/week) exposure to 188 $\mu g/m^3$ NO_2 with superimposed 3 hr/day, 5 days/week peaks of 940 $\mu g/m^3$ NO_2 and 196 $\mu g/m^3$ O_3 mixture resulted in a significant increase in mortality of mice infected with streptococcus aerosol. A similar effect was seen after 9 month exposure to 376 $\mu g/m^3$ NO_2 for 1 hr combined with 196 $\mu g/m^3$ O_3 for 3 hr. The effect, however, was not seen after 4 or 8 month exposure to the same experimental regimen.

Superimposition of a single 3 hr exposure to 0.3 mg/m^3 cupric sulfate at the end of the 9 month exposure to the photochemical oxidant pollutant mixture resulted in further enhancement of the mortality due to the streptococcal pneumonia.

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SECTION 3

RECOMMENDATIONS

Studies should continue to more precisely define the causal relationship between inhalation of air pollutants and acute respiratory infections in laboratory animals.

To simulate environmental pollution conditions studies should include the use of mixtures of gaseous as well as particulate pollutants. Exposures to additional stresses, such as exercise or extremes of temperature also be included in such investigations.

Since man is usually exposed to low concentrations of pollutants with superimposed cyclical higher concentrations, similar exposure regimens should be used during long-term exposure studies. This is especially important in studies of pollutants such as nitrogen dioxide, where within a given exposure regimen the concentration is of greater importance than the duration of exposure.

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SECTION 4

MATERIALS AND METHODS

ANIMALS

The animals used in the various experiments included 3 to 4-week old male and female CD_1 mice (Charles River Breeding Laboratory, Portage, MI) 4 to 8-week old female CD_2F_1 mice (Murphy Laboratory, Plainfield, IN), and male Hartley guinea pigs (Murphy Laboratory). The animals were housed in plastic shoe box cages and quarantined for 7 to 14 days before being used in the studies. Feed (Rodent Laboratory Chow 5001, Ralston Purina Co., St. Louis, MO) and water were available *ad libitum* except during the exposure periods.

During the short-term exposures to the pollutants and to the aerosols of the infectious microorganisms, the animals were held in individual compartments of special stainless-steel wire-mesh cages. For the long term exposures to mixtures of the pollutants the mice were housed in groups of four in inhalation cages and quarantined in the walk-in chambers for 14 days. Feed and water from an automatic watering system were provided *ad libitum* throughout the long-term exposures.

EXPOSURE CHAMBERS

The short-term exposures to the air pollutants were carried out in 432 liter (120x60x60 cm) Plexiglas chambers supplied with filtered room air. The pollutants entered the chamber from the top of one side at a rate of 60 $\pm$ 5 liters/min and were exhausted at the top of the opposite side. To assure a homogeneous distribution of the pollutants, a small fan was operated continuously in each chamber. Deotized cage boards (Upjohn Co., Kalamazoo, MI) were placed on the floor of each chamber to prevent ammonia build-up during the animal exposures. A thermometer and hygrometer (Model 605, Airguide Instrument Co., Chicago, IL) were mounted in each chamber to monitor air temperature and humidity. During the exposures the air temperature in the chambers varied between 23° and 29°C and the humidity from 30 to 50% RH.

For long term exposures to the air pollutant mixtures, 4420 liter (1.2x1.8x2.0m) aluminum-lined walk-in chambers were used. The randomly selected mice were housed in inhalation cages provided with an automatic watering system. To assure unbiased exposure to the experimental atmospheres the cages were rotated weekly to various positions on the cage rack.

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Ammonia build-up during the exposures was prevented by the placement of deotized boards on the dropping pans beneath each cage row. The exposures to the pollutants were interrupted twice weekly for a total of approximately 2 hours to maintain the animals. As part of the maintenance clean cages were provided at bi-weekly intervals, the deotized boards were changed, and fresh feed was provided.

Air flow rates through the walk-in chambers were standardized at approximately 30 changes/hr before the initiation of the exposures and as necessary readjusted at approximately 3 month intervals. The pollutants were diluted and introduced into the chambers in room air which passed through an impregnated activated carbon filter (Type CH, Barnabey and Cheney Co). Shaded-pole blower Model 1C1982A, Dyton Electrical Mfg. Co., Chicago, IL) was continuously operated in each chamber. The distribution of the pollutants within the chambers was calibrated with the blower and pollutant inlet delivery tube in various positions. Once the positions providing the most homogeneous distributions were defined they were used throughout the exposures.

Air temperature and humidity were recorded from a thermometer and precision hygrometer twice daily during the pollutant peak exposures and daily on weekends. Air temperature during the exposure was $22^{o} \pm 3^{o}C$ with extreme excursions ranging from 18^{o} to $28^{o}C$. The humidity varied with ambient and ranged from 20 to 80% RH. The exposure chambers were provided with a manifold system which enabled automatic sampling of the gaseous pollutants. The sampling time for each chamber was set at approximately 15 min.

POLLUTANTS

Nitrogen Dioxide (NO_2)

A 1 or 5% mixture of NO_2 in air (>99.5% pure, Matheson, Joliet, IL) was diluted with filtered air in a mixing chamber and then passed into the exposure chamber. The NO_2 concentration was monitored continuously by a $NO\text{-}NO_2\text{-}NO_x$ chemiluminescent analyzer (Model 8101B, Bendix Corp., Ronceverte, WV) and expressed in ppm and $\mu g/m^3$ (ppm x 1880 = $\mu g/m$).

Ozone (O_3)

A high-voltage generator (IITRI) was used to convert oxygen to O_3. To provide the desired concentration O_3 was diluted with filtered air in a glass mixing chamber and then passed into the exposure chamber. The O_3 concentration was monitored with an O_3 chemiluminescent analyzer (Model OA 310, Meloy Laboratories, Springfield, VA) and expressed in ppm and $\mu g/m$ (ppm x 1960 = $\mu g/m$).

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NO_2-O_3 Mixture

Each gas was introduced into a separate glass vessel and then combined in a glass mixing chamber. The NO_2-O_3 mixture was then passed into the exposure chamber. The concentration of each gas in the exposure chamber was monitored with the chemiluminescent analyzers.

Particulates

The particulate aerosols were generated by a nebulizer (Model 40, DeVilbiss Co., Somerset, PA) from aqueous solutions of crystalline material. The compressed air used to generate and to dilute the aerosol was first passed through an oil filter (Model 170, Deltech Engineering, Inc., New Castle, DE) and an air purifier-flow equalizer (Junior Model, Koby, Inc., Malboro, MA). The flow rates of the compressed air to the nebulizer and the dilution vessel were controlled by needle valves equipped with pressure gauges and tube-float flowmeters. The neck of the nebulizer was connected to a Pyrex glass tube wrapped with a heating tape controlled by a variable autotransformer operated at 80 v (Powerstat, Type 116R, The Superior Electric Co., Bristol, CT). The glass tube was connected to a glass mixing vessel where the particles were diluted with dry filtered air before being introduced into the animal exposure chamber. During a 3-hr exposure the solution in the nebulizer was replenished at 30 min intervals.

The aerosol delivery system was modified somewhat for the multiple exposure studies. The Pyrex tube was replaced by Vycor glass tube, the first 25 cm of which were wrapped with a heating tape controlled by a variable autotransformer set at 410 v.

The estimated mass median diameter of the evaporated sulfate particles was 0.63 μm and the count median diameter approximately 0.31 μm. For particle size analysis, aerosol samples were collected through a 7 μm single-stage preimpactor onto 25 mm glass discs cleaned with methanol and coated with a 50% glycerol-water solution. Microscopic examination indicated that approximately 90% of the airborne particles were <3 μm in diameter. In the multiple exposure studies a piezoelectric quartz crystal microbalance was used periodically to determine the particle-size of the sulfate aerosol. The particulate pollutants included in the studies represented the following sulfates, nitrates and nitrites.

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Sulfates

aluminum sulfate, $Al_2(SO_4)_3 \cdot 18H_2O$, Fisher Scientific Co., Chicago, IL

aluminum ammonium sulfate, $Al_2(SO_4)_3(NH_4)_2SO_4$, Fisher

aluminum zinc sulfate, $Al_2Zn(SO_4)_4$, ICN Pharmaceuticals Inc., Plainfield, NY

ammonium bisulfate, NH_4HSO_4, Fisher

cadmium sulfate, $CdSO_4$, Fisher

cupric sulfate, $CuSO_4$, Baker Chemical Co., Phillipsburg, NJ

ferric sulfate, $Fe_2(SO_4)_3 \cdot nH_2O$, Baker

ferrous sulfate, $FeSO_4 \cdot 7H_2O$, Baker

ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, Anachemica Ltd, Montreal, Que, Canada

magnesium sulfate, $MgSO_4$, Mallinckrodt Chemical, St. Louis, MO

sodium sulfate, Na_2SO_4, Fisher

zinc sulfate, $ZnSO_4$, Baker

Nitrates

aluminum nitrate, $Al(NO_3)_3 \cdot 9H_2O$, Fisher

ammonium nitrate, NH_4NO_3, Baker

cadmium nitrate, $Cd(NO_3)_2 \cdot 4H_2O$, Alfa Division, Danvers, MA

calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$, Fisher

cupric nitrate, $Cu(NO_3)_2 \cdot 3H_2O$, Fisher

lead nitrate, $Pb(NO_3)_2$, Sargent-Welch Co., Skokie, IL

potassium nitrate, KNO_3, Baker

sodium nitrate, $NaNO_3$, Baker

Nitrites

ammonium nitrite, NH_4NO_2, ICN

calcium nitrite, $CaNO_2$, ICN

potassium nitrite, KNO_2, Fisher

sodium nitrite, Na_2NO_2, Baker

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For the quantitative assay of the sulfates or nitrates in the exposure chamber, aerosol samples were collected on membrane filters (Type GS, 0.22 μM, Millipore Corp., Bedford, MA) at a flow rate of 1.5 to 2.0 liters/min. After collection the filters were refluxed with 20 ml of distilled water and the concentration of sulfate was determined by a barium sulfate turbidometric method and expressed as mg/m^3 of sulfate (PHS Publ. No. 999-AP-11, 1965). The nitrate concentration was determined by the 2,4 xyenol method and expressed as mg/m^3 of nitrate (PHS Publ. No. 999-AP-11, 1965). For nitrite analysis aerosol samples were collected with a midget impinger containing 25 ml of absorbing reagent (Ace Glass, Inc., Vineland, NJ). At the end of each hour of exposure, samples were collected for 7 to 12 min at a sampling rate of 1 liter/min. The nitrite concentration was determined by the Saltzman method and expressed as mg/m^3 of nitrite (PHS Publ. No. 999-AP-11, 1965).

INFECTIOUS CHALLENGE

Bacteria

Streptococcus sp. (Lancefield Group C) was used for the respiratory infectious challenge. To maintain the stock culture mice were injected intraperitoneally with a suspension of streptococcus and killed 24 hr later. Heart blood was incubated on blood agar for 48 hr at 37°C and streptococcus colonies isolated from the agar were inoculated in Todd Hewitt broth (BBL). After an 18-hr incubation at 37°C, the bacteria were harvested in approximately 1 ml aliquots and frozen at -70°C. For aerosol dissemination, 0.8 ml of thawed bacteria were grown in Todd Hewitt broth for 18 hr at 37°C. The optical density of the culture was adjusted in 0.1% peptone water to the appropriate density as measured at 440 nm in a Spectronic 20 densitometer.

Alternatively for infectious challenge of CD_1 mice, an aliquot of the stock culture was thawed, inoculated into 100 ml of Todd Hewitt broth, and incubated for 18 hr at 37°C. The culture was centrifuged at 10,000 rpm for 10 min, the supernatant discarded, and the pellet was resuspended in 2 ml of 0.1% peptone water. This suspension was further diluted in peptone water until the desired transmittance (10%) was reached.

Respiratory Challenge

Infectious respiratory challenge was performed in a 400-liter (71x61x92 cm) Plexiglass exposure chamber contained within a microbiological safety cabinet. Temperature and humidity in the chamber was maintained at 24 + 2°C and 65 + 5% RH, respectively. A continuous flow DeVilbiss nebulizer (Model 841) was used to produce the bacterial aerosol. Filtered air was supplied to the inlet of the nebulizer at a rate of 8 liters/min. For infectious challenge mice were housed in individual compartments of stainless steel wire cages. Because of the different innate susceptibility to the infection the CD_2F_1 mice were exposed to the aerosol for 10 min and the CD_1 mice for 30 min. After the challenge the mice were removed from the chamber and housed for a 14-day observation period in an isolated clean-air animal room or were returned to the pollutant exposure chambers.

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The inhaled dose of bacteria was determined by killing three mice from each treatment group immediately after the infectious challenge. The lungs were removed, weighed, and homogenized in 1.8 ml of sterile 0.1% peptone water. The suspension was diluted and plated on blood agar and characteristic streptococcus colonies were counted after 48 hr incubation at 37°C. The inhaled dose generally ranged from 1000 to 2000 viable bacteria per gram of lung tissue.

HEALTH EFFECTS PARAMETERS

Mortality and Survival Time

Deaths were recorded daily during the 14-day observation period following the infectious challenge. Mean survival time was calculated as described by Pindak et al (Proc. Soc. Exp. Biol. Med. 138, 317-321, 1971). The lungs and heart of mice that died after the infectious challenge were aseptically removed and cultured on blood agar plates to confirm the presence of *Streptococcus* sp. At the end of the 14-day observation period the remaining mice were killed and similarly cultured. The blood agar plates were incubated for 48 hr at 37°C.

Bactericidal Activity in the Lungs

Intrapulmonary bacterial inactivation was determined in the lungs of individual mice exposed to radiolabeled live *Klebsiella pneumoniae* aerosol. The ratio of the viable bacterial count to the radioactive count in each lung provided the destruction rate of bacteria by the lung at a given time after infection. *K. pneumoniae* were grown in Anderson's medium in which magnesium sulfate was replaced by magnesium chloride, and the sulfate requirement of the bacteria was provided by addition of ^{35}S-labeled sodium sulfate. After harvest the bacteria were washed and centrifuged ten times at 12,000 xg to remove unattached radiolabel. The concentration of the bacteria was determined by dark field microscopic counts in a Petroff-Hauser chamber and by conventional agar plate culture technique.

After exposure to pollutants, groups of mice were infected by the respiratory route with aerosol of ^{35}S-labeled *K. pneumoniae*. Immediately (0 hr) and at 3 hr after the infectious challenge 10 to 12 mice representing each treatment regimen were killed and their lungs were aseptically removed and homogenized. The total number of bacteria in lungs of each mouse, was calculated from the radioactive counts in aliquots of the lung homogenates and the radioactive labeling ratio determined for each bacterial culture at 0 hr. Based on this information aliquots of each lung homogenate were appropriately diluted to determine by agar plate assay the numbers of viable bacteria that remained in the lungs. The decrease in radioactivity observed during the period between 0 and 3 hr after inhalation of the bacterial aerosol represented the rate of physical removal of the bacteria from the lung.

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Lung Clearance of Inhaled Bacteria

To determine the rate of clearance of inhaled viable bacteria from the lungs, groups of mice representing each pollutant exposure regimen were killed immediately after the respiratory challenge with either *Streptococcus* sp. or *K. pneumoniae* aerosol. The lungs were removed aseptically, weighed and homogenized in 1.8 ml of sterile 0.1% peptone water. The suspension was diluted and plated on blood agar and the colonies were counted after 48 hr incubation at 37°C. These initial counts (0 hr), calculated as the number of viable bacteria per gram of lung, were considered as unity (100%). Thereafter groups of mice were killed at 1,2,3 and 4 hr after the respiratory challenge and their lungs assayed in an identical manner. The counts were calculated as percent recovery of these present at the 0 hr. The rate of clearance was expressed as t_{50} i.e., time in hours required for 50% of the inhaled viable bacteria to be cleared from the lungs. The t_{50} was determined on a semilogarithmic regression after changing the percent recovery to logarithmic values.

In studies of clearance of *Streptococcus* sp. groups of 5 mice were used at each time period. During studies with *K. pneumoniae* 5 mice were also used, with the exception of the 3 hr period where 12 mice were used.

Free Pulmonary Cells

Mice were killed by intraperitoneal injection of sodium pentobarbital and the lungs-heart-trachea complex was removed *in toto*. A blunted hypodermic needle inserted into the trachea and fastened with surgical suture was used to lavage the lungs by repeated infusions of warm saline.

The free pulmonary cells were isolated from the lavage fluids by centrifugation at 365 xg and washing in Hanks medium. Total cell counts were made using a white blood cell diluting pipette and a hemocytometer. To determine the ratio of alveolar macrophages, polymorphonuclear leukocytes and lymphocytes, differential counts were made on air-dried smears stained with Wright's stain. Viability was determined by dye exclusion with 1% trypan blue. At least 200 cells selected ar random were counted microscopically for the differential counts and viability determinations.

Cellular adenosine triphosphate (ATP) concentrations in the lavaged cells were determined using a DuPont 760 Luminescence Biometer. The assay is based on the principle that when a microsample containing ATP is injected into a buffered reaction mixture of luciferase and luciferin, the peak intensity of the resulting light flash is directly proportional to the concentration of ATP. The ATP was extracted from the cells by dimethyl sulfoxide. The extracts were diluted with 0.1 M morpholinopropane sulfonic acid buffer to overcome the quench effect of the high concentration of dimethyl sulfoxide in the aqueous extract for the luciferase-luciferin reaction. ATP levels were expressed as femtograms (10^{-15}g) of ATP per 10^5 AM (calculated from the initial cell counts).

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Lung Edema

To detect edema, groups of 8 to 10 mice were killed and individual lungs weighed immediately after removal. The lungs were then dried in a vacuum dessicator and weighed at 24 hr intervals until no additional weight loss was apparent. The extent of lung edema was expressed as the wet-to-dry weight ratio.

Scanning Electron Microscopy and Histopathology

Mice were anesthetized by intraperitoneal injection of pentobarbital and the abdomen was opened on the midline, permitting access to the ventral aorta. The aorta was severed, the mouse exsanguinated, the chest opened, and the lungs and trachea removed *in toto*. The trachea was cannulated to the level of the first cartilaginous ring, and the lungs were expanded with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative at 20 cm water pressure. After a 2-hr perfusion with the lungs completely immersed in the fixative, the trachea was ligated and the lungs floated in fixative. The trachea and main stem bronchi were isolated from the lungs. The lungs were sectioned with a razor blade to reveal the bronchus and alveoli of each lobe.

All tissues were washed in distilled water and dehydrated with increasing concentrations of alcohol. Pentyl acetate was then substituted for the alcohol, and the tissues were critical-point dried in carbon dioxide. The dried trachea was sectioned longitudinally, and all tissues were cemented to stubs, gold coated in a Denton Vacuum evaporator, and examined in a Kent-Cambridge Mark II Stereoscan scanning electron microscope at 20 kV.

Pulmonary and tracheal tissues were also processed for conventional histopathologic examination. Sections were cut at 5μ, stained with hematoxylin and eosin, and examined microscopically.

Hematology and Clinical Chemistry

In the long term exposure studies, two groups of 10 mice were randomly selected from each chamber, lightly anesthetized with pentobarbital sodium (50 mg/ml), and bled retro-orbitally into either capillary whole blood collectors or blood serum separators (Microtainer 5960, 5961, Becton-Dickinson and Co., Rutherford, NJ). Hematologic parameters evaluated included red blood cell count (RBC x 10^6/mm^3), white blood cell count (WBC x 10^3/mm^3), differential leukocyte count, hemoglobin (Hgb, g/dl) hematocrit (Hct, ml%), mean corpuscular hemoglobin concentration (MCHC, g/dl), mean corpuscular volume (MCV, μ^3) and mean corpuscular hemoglobin (MCH, μμg). With the exception of the differential count where blood smears were examined microscopically, all hematologic measurements were made with an electronic particle counter (Model S, Coulter Electronics, Hialeah, FL).

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Serum levels of selected blood enzymes were determined by microassay with a centrifugal analyzer (Model 500, Centrifi-Chem, Union Carbide, Tarrytown, NY). These enzymes were alkaline phosphatase (modified Bessey-Lowry-Brock technique), lactic dehydrogenase (LDH; lactate pyruvate technique), serum glutamic oxaloacetic transaminase (SGOT; modified Karmen technique), serum glutamic pyruvic transaminase (SGPT; modified Wroblewski and LaDue technique), and creatine phosphokinase (CPK; modified Oliver method). Cholinesterase (Che) was determined manually (Boehringer-Mannheim Diagnostics, Houston, TX).

Statistical Analysis

Treatment differences in mortality rates and mean survival times were evaluated by the Chi-square (X^2) test with 2x2 contingency tables. The Student's t test, linear regression analysis and analysis of variance were applied to the data when appropriate.

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SECTION 5

RESULTS AND DISCUSSION

EXPOSURE TO PARTICULATE AIR POLLUTANTS

The objective of this phase of the program was to determine the effects of inhalation of particulate air pollutants on the resistance to respiratory infection. The pollutants studied during the single and multiple 3-hr exposures represented various sulfates, nitrates and nitrites. Limited experiments were also conducted to determine the effects of exposure to mixtures of sulfate and nitrate aerosols.

The primary health effect parameter observed during these experiments was the mortality rate of mice exposed to the pollutants and challenged with streptococcus aerosol. The effect of inhalation of several particulate air pollutants on the rate of lung clearance of inhaled bacteria and on the pulmonary defense mechanisms in mice were also investigated. In addition, the effects of such exposures were determined in mouse and hamster tracheal organ culture systems.

Effect of Single 3-hr Exposure

Mortality and Survival Time--

Tables 1 and 2 provide an overall summary of experiments in which groups of female CD_2F_1 mice were exposed to at least three concentrations of each sulfate or nitrate and subsequently challenged with the airborne infectious bacteria. In the experiments the duration of the exposure was 3 hr and the concentrations of the particulate pollutants were selected to provide a meaningful dose response for each pollutant. The tables show the concentration of the pollutants expressed in milligrams per cubic meter (mg/m^3) of SO_4 or NO_3, number of mice exposed to each concentration of the pollutants, the number of corresponding control mice exposed to filtered air and the changes in mortality rates and mean survival time. The changes represent the differences between mortality (%) and mean survival rates (days) of mice exposed to the pollutants and those exposed to filtered air.

The differences in mortality rates observed in the experiments were subjected to least square linear regression analyses. Based on these analyses for each pollutant the concentrations were estimated which would produce a 20, 50 or 90% excess mortality over that seen in mice exposed to filtered air. This enabled the ranking of the relative effectiveness

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TABLE 1. CHANGES IN MORTALITY AND SURVIVAL TIME IN MICE EXPOSED FOR 3 HR TO SULFATES AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Exposure Condition		No. of Mice		Change	
Pollutant	mg/m^3	Contr	Exptl	Mortality %	Survival Time, day
$CuSO_4$	0.68	48	48	+ 8.3	- 0.4
	0.87	48	48	+ 27.1*	- 2.2*
	1.07	48	48	+ 68.8*	- 5.9*
	2.31	48	48	+ 91.6*	- 9.2*
	2.58	16	16	+100.0*	-11.3*
$CdSO_4$	0.22	48	46	- 1.2	- 0.1
	0.45	48	48	+ 20.8*	- 1.4*
	0.64	47	48	+ 80.9*	- 9.3*
	1.82	48	47	+ 76.7*	- 7.4*
$Al(SO_4)_3$	0.81	48	47	- 2.0	+ 0.3
	1.20	48	48	0.0	0.0
	1.85	96	96	+ 20.8*	- 1.4*
	2.06	95	96	+ 8.3	- 0.7
	2.36	49	48	+ 29.8*	- 2.6*
$AlZn(SO_4)_4$	1.14	96	98	+ 3.1	- 0.2
	1.37	47	48	- 7.1	+ 0.3
	1.77	48	48	+ 18.7*	- 1.7*
	2.24	49	47	+ 5.4	- 0.7
$Al_2(SO_4)_3(NH_4)_2SO_4$	1.52	47	48	+ 2.1	0.0
	1.77	96	96	+ 5.2	- 0.3
	2.16	96	96	+ 1.0	+ 0.1
	2.45	48	48	+ 8.3	- 0.8
$MgSO_4$	0.73	48	48	- 6.2	+ 0.7
	1.51	96	96	- 3.1	+ 0.3
	2.34	95	96	+ 9.2	- 0.9
$FeSO_4$	1.20	48	47	+ 0.1	+ 0.1
	1.69	49	47	+ 9.0	- 1.0
	2.50	48	48	- 6.3	+ 0.5
	3.40	47	49	- 11.9	+ 1.3

* Significant difference from control mice exposed to filtered air ($p \leq 0.05$).

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TABLE 2. CHANGES IN MORTALITY AND SURVIVAL TIME IN MICE EXPOSED FOR 3 HR TO NITRATES AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Exposure Condition		No. of Mice		Change	
Pollutant	mg/m^3	Control	Exptl	Mortality %	Survival Time, day
$Cu(NO_3)_2$	0.15	95	96	+ 2.0	- 0.3
	0.81	48	48	+33.0*	- 2.2*
	1.05	48	50	+57.4*	- 4.9*
	1.31	48	47	+89.4*	- 8.0*
$Cd(NO_3)_2$	0.18	48	48	- 2.1	0.0
	0.37	48	48	+35.4*	- 2.9*
	0.48	47	48	+ 8.3	- 0.6
	0.97	48	48	+68.7*	- 7.9*
$Al(NO_3)_2$	0.96	48	48	+ 2.1	+ 0.2
	1.76	47	48	+20.7*	- 2.0
	1.88	48	48	- 8.3	+ 0.9
	1.97	48	48	+14.6*	- 1.1*
	2.14	48	48	+56.2*	- 5.5*

* Significant difference from control mice exposed to filtered air ($p \leq 0.05$).

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of the various particulate pollutants in altering the resistance to streptococcal pneumonia. As seen in Table 3, cadmium sulfate and cadmium nitrate were the most toxic particulate pollutants among those included in the studies. In decreasing order of toxicity were copper, aluminum and magnesium sulfate or nitrate aerosols. The similar response after inhalation of sulfate or nitrate pollutants confirmed the previously reported observation that the metallic cation is primarily responsible for the enhanced susceptibility to the bacterial respiratory infection. The importance of the cation in altering the host defense mechanism could be seen more clearly when the aerosol concentrations of the pollutants were recalculated and expressed as mg/m^3 of the metallic cation. Exposure to copper and cadmium produced 20% excess mortality when their aerosol concentration ranged from 0.22 and 0.39 mg/m^3. This was irrespective whether or not these compounds were in the form of sulfate or nitrate aerosol. The concentration of magnesium necessary to produce a similar enhancement in mortality was 0.88 mg/m^3. The aerosol concentration of aluminum estimated to be required to produce a 20% excess mortality ranged from 0.23 mg/m^3 for aluminum nitrate, 0.39 mg/m^3 for aluminum sulfate, 0.48 mg/m^3 for aluminum zinc sulfate to 2.18 mg/m^3 for aluminum ammonium sulfate. The higher aerosol concentration of aluminum in aluminum ammonium sulfate necessary to enhance the mortality could be due to the presence of the ammonium ion which could possibly neutralize the effects of the metallic cations.

Table 4 shows a number of other particulate pollutants and their highest aerosol concentration used in the experiments which after a 3-hr exposure, did not result in any significant changes in mortality rate or survival time.

Pulmonary Defenses--

The effects of inhalation of sulfate aerosols on pulmonary defenses were evaluated by examining free cells obtained by tracheobronchial lavage and by measuring pulmonary bactericidal activity in the lung. The number of total cells recovered from lungs of mice exposed to cupric sulfate or aluminum sulfate was significantly lower when compared to the number of cells lavaged from mice exposed to filtered air (Table 5). However, the viability or the ATP levels of alveolar macrophages lavaged immediately after exposure to cupric or aluminum sulfate aerosols did not differ from the controls. Exposure to aluminum ammonium sulfate had no effect on the total cell count or the alveolar macrophages. Differential cell counts, not shown in the table, were not affected by the exposures to any of the three sulfates.

The bactericidal activity in the lungs was determined in mice challenged with ^{35}S-labeled *K. pneumoniae* aerosol within 1 hr after exposure to the sulfates. The rate of killing and physical removal of the bacteria from the lungs was determined 3 hr after inhalation of the radiolabeled bacteria (Table 6). When compared to the corresponding control mice the percent of bacteria killed significantly increased in lungs of mice exposed to aluminum sulfate but significantly decreased in those exposed to aluminum

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TABLE 3. RELATIVE EFFECTIVENESS OF A 3 HR EXPOSURE TO VARIOUS PARTICULATE POLLUTANTS IN ALTERING THE RESISTANCE TO STREPTOCOCCAL PNEUMONIA

Exposure Condition Pollutant	Conc Range mg/m^3	No. of Expts	SO_4 or NO_3 mg/m^3 Producing Excess Mortality of 20%	50%	90%
$Cu(NO_3)_2$	0.15 - 1.31	5	0.48	0.90	1.45
$CuSO_4$	0.68 - 2.58	6	0.58	1.30	2.25
$Cd(NO_3)_2$	0.18 - 0.97	4	0.41	0.77	1.26
$CdSO_4$	0.22 - 1.82	7	0.19	0.93	1.91
$Al(NO_3)_2$	0.96 - 2.14	5	1.62	2.49	3.66
$Al_2(SO_4)_3$	0.81 - 2.36	8	2.05	3.62	5.70
$Al_2Zn(SO_4)_4$	1.14 - 2.24	5	3.42	7.06	11.9
$Al_2(SO_4)_3(NH_4)_2SO_4$	1.52 - 2.45	7	15.6	40.5	73.7
$MgSO_4$	0.73 - 2.34	4	3.52	6.26	9.91
$FeSO_4$	1.20 - 3.40	8	negative dose-response		
NH_4HSO_4	4.42 - 6.66	3	negative dose-response		

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TABLE 4. HIGHEST TESTED AEROSOL CONCENTRATION OF PARTICULATE POLLUTANTS WHICH DID NOT ALTER THE RESISTANCE TO RESPIRATORY INFECTION

Pollutant	Conc., mg/m^3
Ferric sulfate $Fe_2(SO_4)_3$	2.90
Ferric ammonium sulfate $Fe(NH_4)_2(SO_4)_2$	2.23
Sodium sulfate Na_2SO_4	4.04
Sodium nitrate $NaNO_3$	3.12
Potassium nitrate KNO_3	4.30
Ammonium nitrate NH_4NO_3	4.50
Lead nitrate $Pb(NO_3)_2$	2.05
Calcium nitrate $Ca(NO_3)_2$	2.75
Ammonium nitrite NH_4NO_2	4.57
Potassium nitrite KNO_2	4.51
Calcium nitrite $CaNO_2$	3.74
Sodium nitrite Na_2NO_2	4.73

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TABLE 5. EFFECT OF A 3 HR EXPOSURE TO SULFATES ON THE FREE CELLS LAVAGED FROM LUNGS OF MICE

Exposure Condition		Total Cells ($x10^5$)		Alveolar Macrophages			
				Viability, %		ATP$x10^8$ (fg/10^5AM)	
Pollutant	mg/m^3	Mean	SE	Mean	SE	Mean	SE
Control	0	6.93	0.38	98.2	0.2	1.02	0.05
$CuSO_4$	1.30	4.27*	0.26	98.1	0.3	0.92	0.05
Control	0	6.10	0.41	97.4	0.3	0.99	0.06
$CuSO_4$	2.58	3.92*	0.33	97.8	0.2	1.04	0.07
Control	0	4.75	0.37	97.6	0.3	1.07	0.06
$Al_2(SO_4)_3$	2.42	3.50*	0.37	98.2	0.2	1.12	0.14
Control	0	4.46	0.27	97.2	0.3	0.92	0.05
$Al_2(SO_4)_3(NH_4)_2SO_4$	2.19	4.66	0.27	98.3	0.2	0.87	0.05

* Significant difference from corresponding control mice exposed to filtered air ($p \leq 0.05$).

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TABLE 6. EFFECT OF A 3 HR EXPOSURE TO SULFATES ON THE BACTERICIDAL ACTIVITY IN LUNGS OF MICE

Exposure Condition Pollutant	mg/m^3	Bacteria Killed, %[a] Mean	SE	Physical Removal, %[b] Mean	SE
Control	0	81.3	4.5	28.4	2.4
$CuSO_4$	2.30	79.0	2.3	34.7	2.9
Control	0	85.5	1.7	27.9	2.9
$Al_2(SO_4)_3$	1.47	91.8*	1.0	14.9*	1.9
Control	0	90.9	1.4	42.5	2.0
$Al_2(SO_4)_3(NH_4)_2SO_4$	3.76	84.6*	1.8	32.4*	3.2

[a] Percent of ^{35}S-*K. pneumoniae* killed in lungs 3 hr after inhalation.

[b] Percent of physical removal of ^{35}S-*K. pneumoniae* from lungs 3 hr after inhalation.

* Significant difference from corresponding control mice exposed to filtered air ($p \leq 0.05$).

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ammonium sulfate. The percent inhaled bacteria physically removed by the lungs was significantly reduced after inhalation of 1.5 mg/m^3 $Al_3(SO_4)_3$ or 3.8 mg/m^3 $Al_2(SO_4)_3(NH_4)_2SO_4$. Exposure to cupric sulfate had no significant effect on any of the two indices of pulmonary defenses.

Effect on Tracheal Epithelium--

The effects of cupric sulfate, aluminum sulfate, and aluminum ammonium sulfate on ciliary activity and structure of the tracheal epithelium was determined in hamster and mouse tracheal organ cultures. The parameters used to measure these effects were the cilia beating frequency and morphological and cytopathological changes.

Male Syrian golden hamsters 4 weeks old and female CDF_1 mice 4 to 6 weeks old were exposed for 3 hr to aerosols of the particulate pollutants, while control animals were exposed to filtered ambient air. Immediately after the exposure the tracheas were removed, cut transversely into a series of rings and the alterations in beating frequency of the cilia and cytopathological changes were observed.

The effects of the various sulfates on cilia beating frequency and the percent of normal epithelium are shown in Table 7. A decrease in beating frequency was seen in mice but the statistical significance could not be ascertained. A significant ($p \leq 0.05$) decrease in the mean beating frequency of cilia was seen in tracheas of hamsters exposed for 3 hr to any of the three sulfates. The tracheal epithelium of hamsters exposed to the sulfates showed significant cytopathological changes. In control hamsters the mean percent of normal epithelium, defined as smooth luminal surface with beating cilia, ranged from 85 to 93%, whereas after exposure to the sulfates the normal epithelium areas ranged from 59 to 68%. A similar significant decrease in normal epithelium was seen only in mice exposed for 3 hr to aluminum sulfate. Since the control CDF_1 mice exposed to filtered air showed only approximately 56% of normal epithelium, this strain of mice does not appear to be the appropriate model for the assessment of this parameter.

Histopathological examination of tracheas revealed no discernible differences between mice exposed to sulfate aerosols and control mice exposed to filtered air. Exfoliation of differentiated surface epithelial cells from the mucosa was frequently seen in all mice but the extent of desquamation was markedly higher in mice exposed to the sulfates. Cytoplasmic vacuolization was observed in approximately 10% of the epithelium of mice exposed to the sulfates. Tracheas of hamsters examined after exposure to cupric or aluminum sulfate did not differ markedly from those of hamsters exposed to air. Occasional changes seen were moderate epithelial flattening and focal areas of exfoliation of surface epithelial cells. These changes were probably responsible for the significant alterations seen during the cytological examinations of the unfixed tracheal epithelium.

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TABLE 7. MEAN CILIA BEATING FREQUENCY AND PERCENT OF NORMAL EPITHELIUM IN HAMSTER AND MOUSE TRACHEAS IMMEDIATELY AFTER 3 HR EXPOSURE TO SULFATES

Exposure Condition		Cilia Beat/min[a]				Normal Epithelium, %[b]			
		Mouse		Hamster		Mouse		Hamster	
Pollutant	mg/m³	Air	SO_4	Air	SO_4	Air	SO_4	Air	SO_4
$CuSO_4$	2.30	1295	1242	1238	1153*	55	43	86	62*
$Al_2(SO_4)_3$	1.47	1290	1251	1232	1175*	56	28*	85	68*
$Al_2(SO_4)_3(NH_4)_2SO_4$	3.76	1251	1234	1216	1143*	56	54	93	59*

[a] Mean of 48 observations of 12 tracheal rings from four animals.

[b] Mean of 12 tracheal rings from four animals.

* Significant difference from corresponding control animals exposed to filtered air ($p \leq 0.05$).

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Effect of Interval Between Exposure and Challenge

Limited experiments were conducted to determine the persistence of changes in susceptibility to the respiratory infection induced by inhalation of cupric sulfate or zinc sulfate aerosols. In one experiment groups of 24 mice were exposed for 3 hr to the particulate aerosols at 168, 72, 24 or <1 hour before the infectious challenge. Mice representing each treatment and control mice exposed to filtered air, were then pooled and together challenged with the streptococcus aerosol. In another type of experiment all mice were simultaneously exposed to the particulate pollutants and then, 168, 72, 24 or <1 hour later, were challenged with infectious aerosols. A group of mice exposed to filtered air served as a control in each infectious aerosol challenge.

Results of these studies are summarized in Table 8. The increased susceptibility to the infection after inhalation of 0.6 mg/m^3 $CuSO_4$ aerosol was observed when the interval between the exposure and the infectious challenge was less than 1 hour; this effect was not seen when the interval was extended to 24 hrs or longer. After exposure to 1.35 mg/m^3 $CuSO_4$ significant increased mortality when compared to controls persisted for at least 72 hrs. A marked increase in mortality (12.5%) was also seen 7 days (168 hr) after the exposure but the statistical significance of this increase could not be ascertained. The effect of exposure to 2.4 and 2.5 mg/m^3 $ZnSO_4$ persisted for up to 72 hrs at which time the significant increases in mortality rates were 35.9 and 29.2%. However, this effect was totally reversed at 7 days after the exposure to this pollutant, when a significant decrease in mortality (37.5%) was seen.

Exposure to Mixtures of Particulate Pollutants

Exploratory experiments were conducted to determine the effect of exposure to mixtures of particulate pollutants. The changes in mortality rates in mice challenged with streptococcus aerosol after a single 3-hr exposure to cupric sulfate and sodium nitrate mixture of cupric sulfate and cupric nitrate mixture are shown in Table 9. The expected excess mortalities for mice exposed to the given concentration of the individual pollutants were calculated from the least-square line of regression models obtained from previous studies of these pollutants. When the estimated changes in mortality rate for the individual pollutants were added and compared to those observed after actual exposures to the mixtures, a good agreement between the two values was apparent. Thus, the effects of the pollutants were additive and, within the limits of these studies, no indication of a synergism between the pollutants was seen.

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TABLE 8. EFFECT OF INTERVAL BETWEEN EXPOSURE TO PARTICULATE POLLUTANTS AND INFECTIOUS CHALLENGE ON MORTALITY AND SURVIVAL TIME

Exposure Condition		Interval Between Exposure and Challenge (hr)							
		Mortality Change, %				Survival Change, days			
Pollutant	mg/m³	1	24	72	168	1	24	72	168
$CuSO_4$	0.60	+23.6	0	0	-	-1.5*	-0.2	+0.1	-
	1.35	+33.3*	+45.8*	+25.0*	+12.5	-3.2*	-4.0*	-2.1*	-0.8
$ZnSO_4$	2.39	+87.5*	+20.9*	+35.9*	-37.5*	- 8.9*	-1.9*	-5.2*	+3.0*
	2.49	+62.5	+54.2*	+29.2*	-37.5*	- 7.8*	-5.8*	-2.6*	+3.1*

* Significant difference from control mice exposed to filtered air ($p \leq 0.05$).

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TABLE 9. ESTIMATED AND ACTUAL CHANGES IN MORTALITY RATE OF MICE EXPOSED FOR 3 HR TO A MIXTURE OF SULFATE AND NITRATE POLLUTANTS AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Exposure Condition			Mortality Change, %[a]		
Pollutant Mixture	Conc mg/m³ SO_4/NO_3	Conc mg/m³ Metal	Expected[b] Single	Expected[b] Mixture	Actual Mixture
$CuSO_4$	0.39	0.26	+11.9		
$NaNO_3$	3.46	1.28	+ 7.2	+19.1	+ 4.2
$CuSO_4$	1.03	0.68	+39.0		
$NaNO_3$	3.02	1.12	+ 6.0	+45.0	+52.1*
$CuSO_4$	0.46	0.30	+14.9		
$CuNO_3$	0.16	0.08	- 5.6	+ 9.3	+10.4
$CuSO_4$	0.62	0.41	+21.7		
$CuNO_3$	0.42	0.21	+12.9	+34.6	+37.5*

[a] Pollutant-Control, based on 48 mice/treatment.

[b] Estimated from least-squares linear regression equations.

* Significant difference from corresponding control mice exposed to filtered air ($p \leq 0.05$).

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Effect of Multiple 3-hr Exposures

For the intermittent exposure studies mice were exposed to either cupric sulfate or zinc sulfate for 3 hr/day, 5 days/week for 1, 2 and 4 weeks. Within 1 hr following the final exposure the mice were challenged with the streptococcus aerosol and the mortalities were recorded during the 14-day post-infection observation period. The aerosol concentration of both pollutants was approximately 0.1 mg/m^3 and the mass median diameter (MMD) of the aerosol particles was approximately 0.8 μm. During the 3-hr exposures the air temperature and humidity in the chambers averaged 26°C and 45% RH.

Mortality rates were not affected by 5 or 10 daily exposures to either of the two pollutants (Table 10). However, 20 daily 3-hr exposures to zinc sulfate or cupric sulfate resulted in significant (+13.9 and +20.9%) increases in mortality rates. The rate of clearance of the inhaled bacteria from the lungs (t_{50}) and the percent of inhaled bacteria killed in lungs were also determined. The summary of data in Table 11 indicates that in general the differences between the controls and experimental treatments were not statistically significant. However, an almost twofold increase in t_{50} was seen after 20 repeated exposures to zinc sulfate and 5, 10 or 20 exposures to the cupric sulfate aerosol. The 20 daily exposures to cupric sulfate resulted in a significant decrease ($p \leq 0.1$) in the percent of inhaled bacteria killed in the lungs (84.6% control vs. 76.7% experimental).

EXPOSURES TO NO_2 and O_3 MIXTURES

Two experiments were conducted to determine the effects of long term exposures to various mixtures of NO_2 and O_3 on the resistance to respiratory infection. The differences between these two studies were the concentrations of the pollutants, the exposure regimens, and housing and husbandry of the animals. In the first study the animals were exposed to a continuous background concentration of NO_2 with a superimposed single daily 3-hr peak of a NO_2 or a NO_2 and O_3 mixture. In the second study two daily peaks and higher concentrations of NO_2 were used.

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TABLE 10. MORTALITY RATE OF MICE REPEATEDLY EXPOSED FOR 3 HR TO SULFATES AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Pollutant	Exposure Condition: No. of Exp	SO_4 mg/m³: Mean	SO_4 mg/m³: SD	Mortality,%[a]: Control	Mortality,%[a]: Exptl	Mortality,%[a]: Change
$ZnSO_4$	5	0.10	0.013	19.4	22.2	+ 2.8
	10	0.10	0.011	12.5	11.1	- 1.4
	20	0.10	0.011	54.2	68.1	+13.9*
$CuSO_4$	5	0.10	0.042	2.1	2.1	0
	10	0.11	0.030	4.2	4.2	0
	20	0.10	0.036	20.8	41.7	+20.9**

Significant difference from corresponding control mice exposed to filtered air (*p $\leq$0.1 **p $\leq$0.05).

[a]Based on 72 mice/group for $ZnSO_4$ and 48 mice/group for $CuSO_4$ exposures.

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TABLE 11. EFFECT OF MULTIPLE 3 HR EXPOSURES TO SULFATES ON THE PULMONARY DEFENSE MECHANISMS

Pollutant	No. of Exp[a]	Experimental Parameter: t_{50} (hr) Control	t_{50} (hr) Exptl	% Bact Killed Control	% Bact Killed Exptl	log PFC/10^6 Cells Control	log PFC/10^6 Cells Exptl
$ZnSO_4$	5	1.05	1.07	91.0	92.8	3.41	3.35
	10	0.89	0.76	86.7	83.1	3.56	3.53
	20	0.62	1.09	91.2	91.5	3.48	3.65
$CuSO_4$	5	0.74	1.32	85.3	83.1	3.47	3.42
	10	1.07	1.68	79.4	80.3	3.52	3.56
	20	0.82	1.20	84.6	76.7*	N.D.	N.D.

Significant difference from corresponding control mice exposed to filtered air (*$p \leq 0.1$).

[a]See Table 10 for SO_4 concentrations.

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Continuous Exposure with Single Daily Peak

The environmental exposure conditions used in this first study were as follows:

Exposure Designation	Continuous Exposure 24 hr/day, 7 d/week	Peak Exposure 3 hr/day, 5 d/week
O	Filtered Air	None
$O(NO_2)$	Filtered Air	940 $\mu g/m^3 NO_2$
$O(NO_2+O_3)$	Filtered Air	940 $\mu g/m^3 NO_2$ and 196 $\mu g/m^3 O_3$ mixture
$NO_2(NO_2+O_3)$	188 $\mu g/m^3 NO_2$	940 $\mu g/m^3 NO_2$ and 196 $\mu g/m^3 O_3$ mixture
$O(O_3)$	Filtered Air	196 $\mu g/m^3 O_3$

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Female CD_2F_1 mice were housed in groups of 8 in large wire mesh cages and after a 14 day quarantine period were exposed to the various air pollutant regimens in the same type of cages. After 1, 3 and 6 months exposure, groups of mice were challenged with streptococcus aerosol and returned to the respective environment for a 14 day post-infection and observation period. The data shown in Table 12 indicate that significant increases in the mortality rates were seen only after 6 month exposure to the various pollutant regimens. The changes in mortality rates observed after 1 and 3 month exposure were not significant.

In addition to the mice designated for the infectious challenge and mortality studies, groups of 10 mice and 4 guinea pigs were housed in the environmental chambers. These animals were used to determine changes in body weights and in selected blood serum parameters during the exposures. In general no significant changes related to the exposures were seen in body weights, serum cholinesterase, SGPT or LDH in mice. Similarly 1, 3 and 6 month exposure no significant changes were seen in the body weights, serum cholinesterase, acid phosphatase, CPK, SGOT, SGPT and LDH in guinea pigs.

Continuous Exposure with Two Daily Peaks

The health effects observed during the second long term study included mortality and survival rates after challenge with streptococcal aerosol, clearance rate of inhaled viable bacteria from the lungs, pulmonary bactericidal activity and changes in pulmonary free cells. In addition, body weight and various hematological and clinical pathology parameters were determined.

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TABLE 12. MORTALITY RATES OF MICE EXPOSED TO NO_2 AND O_3 MIXTURES AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Exposure Regimen	Mortality Rate								
	1 month			3 month			6 month		
	D/T	%	Change	D/T	%	Change	D/T	%	Change
0	21/93	22.6	-	25/135	18.5	-	7/72	9.7	-
$0(NO_2)$	23/95	24.2	+1.6	26/125	20.8	+2.3	15/72	21.8	+11.1*
$0(NO_2+O_3)$	19/95	20.0	-2.6	30/135	22.2	+3.7	15/72	21.8	+11.1*
$NO_2(NO_2+O_3)$	15/96	15.6	-7.0	34/135	25.2	+6.7	16/72	22.2	+12.5**
$0(O_3)$	17/96	17.7	-4.9	22/133	16.5	-2.0	14/72	19.4	+ 9.7*

Significant difference from corresponding control mice exposed to filtered air
*p $\leq$0.1 **p $\leq$0.05.

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The four exposure regimens used in the second study were as follows:

Exposure Designation	Continuous Exposure 24 hr/day, 7 d/week	Peak Exposure 5 days/week	
0	Filtered Air	AM	None
		PM	None
NO_2	376 $\mu g/m^3$ NO_2	AM	None
		PM	None
$NO_2(NO_2)$	376 $\mu g/m^3$ NO_2	AM	1880 $\mu g/m^3$ NO_2/1 hr
		PM	1880 $\mu g/m^3$ NO_2/1 hr
$NO_2(NO_2+O_3)$	376 $\mu g/m^3$ NO_2	AM	1880 $\mu g/m^3$ NO_2/1 hr
		PM	196 $\mu g/m^3$ O_3/3 hr mixed with 1880 $\mu g/m^3$ NO_2/hr

Female CD_1 mice, 21 to 28 days old were housed in groups of four in the individual compartments of the inhalation cages and held in the environmental exposure chambers. After 7 and 14 days 15 mice were randomly selected for microbiologic monitoring and gross pathological examination. Gross necropsy revealed no lesions, and nasal, tracheal, lung or fecal cultures were negative for pathogenic microorganisms and ecto- and endoparasites.

At the end of the 14 day quarantine period the mice were randomly assigned to the individual compartments of the inhalation cages. Two mice were housed in each of the 288 compartments of a cage rack. Each cage rack was then randomly assigned to an exposure chamber providing the four environmental conditions. From each cage rack 24 randomly selected mice were stratified by weight, individually identified and were used for body weight determinations throughout the exposure. Otherwise mice were randomly selected and assigned to groups for studies of the various health parameters.

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Mortality--

At the completion of 4, 8 and 9 month mice representing each of the air pollutant exposure regimens were challenged with the streptococcus aerosol. After the infectious challenge the mice were returned to the respective environmental chambers and the mortality was recorded during the ensuing 14-day observation period. The data summarized in Table 13 indicate that in general the exposures had no significant effect on the mortality rate resulting from the infectious challenge. The only significant ($p \leq 0.1$) increase in mortality rate was seen after 9 month exposure to 376 $\mu g/m^3$ NO_2 with the superimposed daily NO_2 and O_3 mixture peaks. The mean survival time reflected the pattern of mortality rates.

Clearance Rates and Bactericidal Activity--

The clearance rate of inhaled viable bacteria from lungs was determined after 4 and 8 month exposure to the pollutants. At the same time periods the bactericidal activity in the lungs was also assessed. The clearance rate was estimated by determining the concentration of *K. pneumoniae* in the lungs immediately after and at 1, 2, 3, and 4 hrs after the respiratory challenge. The percent recoveries were converted to logarithms and the least squares linear regression analysis was used to estimate the t_{50} value and the regression coefficient. An approximately two-fold increase in the t_{50} was seen in mice exposed for 4 or 8 months to NO_2 with the superimposed daily NO_2 peaks as compared to control mice exposed to filtered air (Table 14). On the other hand, when compared to the corresponding control, continuous exposure to NO_2 only resulted in a decrease in the t_{50} value. None of the differences in t_{50} were, however, statistically significant. The significance of the differences between the regression coefficients (b) of the linear regression lines of the controls and those of mice exposed to the air pollutant regimens were determined by use of the Student t test. After the 8 month exposure the difference between regression coefficients of control mice (-0.31) and those continuously exposed to NO_2 (-0.22) and controls and those exposed to NO_2 with the superimposed NO_2 and O_3 peaks (-0.20) were significant (t 1.73 and 2.12).

The percent of inhaled *K. pneumoniae* killed in the lungs at 3-hr after the respiratory challenge with the infectious bacterial aerosol is also shown in Table 14. A significant ($p \leq 0.05$) decrease in bactericidal activity, was seen in mice exposed for 8 months to the continuous NO_2 regimen when compared to the control mice exposed to filtered air.

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TABLE 13. MORTALITY RATES OF MICE EXPOSED TO NO_2 AND O_3 MIXTURES AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Exposure Regimen	Mortality Rate								
	4 month			8 month			9 month		
	D/T	%	Change	D/T	%	Change	D/T	%	Change
0	29/72	40.3	-	5/48	10.4	-	11/62	17.7	-
NO_2	25/72	34.7	-5.6	6/48	12.5	+2.1	12/71	16.9	-0.8
$NO_2(NO_2)$	28/72	38.9	-1.4	4/48	8.3	-2.1	17/70	24.3	+ 6.6
$NO_2(NO_2+O_3)$	27/72	37.5	-2.8	5/48	10.4	0	20/64	31.3	+13.6*

Significant difference from corresponding control mice exposed to filtered air
*p $\leq$0.1.

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TABLE 14. RATE OF CLEARANCE OF VIABLE *K. PNEUMONIAE* FROM LUNGS OF MICE EXPOSED TO NO_2 AND O_3 MIXTURES

Exposure Regimen	Clearance Rate				Bactericidal Activity (%)[a]	
	4 mo		8 mo		4 mo	8 mo
	t_{50}[b]	b[c]	t_{50}	b		
0	1.05	-0.25	0.54	-0.31	59.5	88.6
NO_2	1.28	-0.26	0.23	-0.22*	71.0	68.5*
$NO_2(NO_2)$	2.38	-0.18	0.89	-0.23	66.5	90.8
$NO_2(NO_2+O_3)$	1.12	-0.26	0.45	-0.20**	68.3	81.8

[a] Percent of inhaled bacteria killed at 3 hr after challenge.

[b] Time (hr) required to clear 50% of inhaled bacteria.

[c] Regression coefficient.

Significant difference from control mice exposed to filtered air
*$p \leq 0.1$ **$p \leq 0.05$.

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Pulmonary Cells--

The concentration of free pulmonary cells obtained by lavage and the ATP level of alveolar macrophages were determined after 4 and 8 month exposure to the pollutants (Table 15). No significant changes in total free pulmonary cell counts were detected in lung lavages obtained within 1 hr of termination of the 4 or 8 month exposures to the pollutants. Significant increases of 25% and 35% in cellular ATP level were seen after 4 and 8 months exposure to NO_2 with the superimposed daily peaks of NO_2 and O_3 mixture.

Hematology and Clinical Pathology Parameters--

After the 4 and 8 month exposures groups of 10 mice representing each exposure regimen were killed and blood samples were submitted to hematological and clinical pathology examinations. The results of the hematological examinations shown in Table 16 indicate that after 4 months the differences between the control values and those of the exposure regimens were not significant for any of the parameters. After 8 months a significant ($p \leq 0.05$) increase in erythrocytes, leukocytes and hematocrit, and a significant decrease in mean corpuscular hemoglobin concentration (MCHC) was seen in mice exposed to NO_2 with superimposed NO_2 and O_3 mixture peaks when compared to control mice exposed to filtered air. The results of blood serum enzyme level assays summarized in Table 17 indicate some significant differences related to the environmental exposures. The experimental regimen involving continuous NO_2 exposure with superimposed NO_2 and O_3 mixture peaks appeared to be most effective in inducing these changes.

The body weights did not differ significantly and the overall increase during the 8 month experimental period ranged from 34 to 38%. At the completion of the exposure the mean body weight of control mice was 32.3 g and of those exposed to the various pollutant regimens it was 31.3, 31.8 and 31.9 g.

EXPOSURE TO OXIDANT AND PARTICULATE POLLUTANT MIXTURES

The decision to terminate the long term exposures to NO_2 and O_3 mixtures after 8 months provided the opportunity to use the remaining mice for interaction studies with particulate pollutants. After 9 months groups of mice maintained at each of these NO_2 and O_3 exposure regimens and appropriate controls were exposed for a single 3-hr period to 0.1 mg/m^3 or 0.3 mg/m^3 $CuSO_4$ aerosols. Within less than 1 hour later the mice were challenged with Streptococcus aerosol and the mortalities were recorded during the ensuing 14 day observation period. The observed mortality rates (Table 18) were examined using the Chi-square analysis to determine the significance of the differences. In general, the data indicate a close relationship between the concentration of the sulfate aerosol and the mortality rate. With the exception of mice exposed continuously to NO_2,

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TABLE 15. EFFECT OF EXPOSURE TO NO_2 AND O_3 MIXTURES ON FREE PULMONARY CELLS

Exposure Regimen	Pulmonary Cells[a]				Alveolar Macrophage(ATP)[b]			
	4 month		8 month		4 month		8 month	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	3.55	.33	3.23	.40	0.77	.04	0.75	.08
NO_2	3.37	.44	3.89	.36	0.80	.07	0.66	.07
$NO_2(NO_2)$	4.15	.37	3.81	.46	0.83	.09	0.60	.06
$NO_2(NO_2+O_3)$	3.67	.35	2.82	.31	0.96*	.08	1.01*	.12

[a] Total cell counts ($x10^5$)

[b] Cellular ATP ($10^8 fg/10^5$ cells)

Significant difference from corresponding controls exposed to filtered air (*$p \leq 0.1$).

TABLE 16. HEMATOLOGICAL VALUES OF MICE EXPOSED TO NO_2 AND O_3 MIXTURES

Exposure Regimen	RBC, $\times 10^6$ ml		Hgb, g/dl		Hct, %		MCV, μm^3		MCH, $\mu\mu g$		MCHC, g/dl	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
4 month												
0	8.2	0.3	15.4	0.6	39.3	1.5	48.8	0.5	19.1	0.1	39.8	0.3
NO_2	8.8	0.3	16.3	0.4	41.5	1.2	47.8	0.6	18.7	0.3	39.8	0.3
$NO_2(NO_2)$	8.4	0.2	15.8	0.5	40.0	1.1	48.8	0.5	19.2	0.3	39.9	0.3
$NO_2(NO_2+O_3)$	8.7	0.3	16.1	0.7	41.3	1.7	48.7	0.6	18.9	0.2	39.6	0.3
8 month												
0	8.3[a]	0.2	16.1	0.3	39.1[a]	0.7	48.0	0.7	19.8	0.3	42.2[a]	0.5
NO_2	8.3[ab]	0.2	16.0	0.3	39.6[a]	1.4	48.7	0.5	19.7	0.2	41.1[ab]	0.6
$NO_2(NO_2)$	8.4[ab]	0.2	16.1	0.3	40.7[a]	1.1	49.0	0.7	19.8	0.3	40.1[b]	0.5
$NO_2(NO_2+O_3)$	8.9[b]	0.2	16.7	0.4	42.0[b]	0.8	48.6	0.8	19.2	0.3	40.2[b]	0.6

Table 16 continued.

TABLE 16 (continued)

Exposure Regimen	WBC x10^3/ml Mean	SE	Neutroph x10^3/ml Mean	SE	Lympho x10^3/ml Mean	SE	Monocyt x10^3/ml Mean	SE
4 month								
0	4.2	0.6	0.51	0.12	3.6	0.5	0.06	0.02
NO_2	3.8	0.4	0.79	0.14	3.0	0.4	0.07	0.02
$NO_2(NO_2)$	3.6	0.4	0.57	0.10	3.0	0.3	0.07	0.01
$NO_2(NO_2+O_3)$	4.1	0.8	0.59	0.13	3.2	0.6	0.09	0.04
8 month								
0	3.0[a]	0.3	0.46[a]	0.08	2.5[a]	0.3	NOT DONE	
NO_2	4.2[b]	0.4	0.86[c]	0.08	3.3[ab]	0.4		
$NO_2(NO_2)$	4.6[b]	0.9	0.73[bc]	0.10	3.9[ab]	0.8		
$NO_2(NO_2+O_3)$	4.2[b]	0.9	0.64[ab]	0.06	3.5[b]	0.4		

Values within each column with the same superscript are not significantly different ($p \leq 0.05$).

TABLE 17. SERUM ENZYME LEVELS (IU/liter) IN MICE EXPOSED TO NO_2 AND O_3 MIXTURES

Exposure Regimen	Che		Alk Phosph		CPK		SGOT		SGPT		LDH	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
4 month												
0	4159[ab]	183	38	4	303	44	64[ab]	4	17	1	296	16
NO_2	3852[a]	215	42	3	301	48	64[ab]	5	18	2	336	32
$NO_2(NO_2)$	4320[b]	135	48	5	307	39	69[b]	5	19	1	302	23
$NO_2(NO_2+O_3)$	4464[b]	98	48	3	328	39	58[a]	2	16	1	278	15
8 month												
0	10358	271	51	5	331[a]	55	98	8	18	2	388	31
NO_2	11035	554	45	4	187[b]	20	84	6	19	2	385	61
$NO_2(NO_2)$	9932	451	51	5	230[ab]	36	89	6	18	2	397	40
$NO_2(NO_2+O_3)$	11348	557	53	6	248[ab]	58	104	16	20	2	491	84

Values within each column with the same superscript are not significantly different ($p \leq 0.05$).

TABLE 18. MORTALITY RATE OF MICE EXPOSED FOR 9 MONTHS TO NO_2 AND O_3 MIXTURES, ONCE FOR 3 HR TO $CuSO_4$ AEROSOL AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

$CuSO_4$ mg/m^3	NO_2 and O_3 Exposure Regimen							
	0		NO_2		$NO_2(NO_2)$		$NO_2(NO_2+O_3)$	
	D/T	%	D/T	%	D/T	%	D/T	%
0	11/62	17.7[a]	12/71	16.9[a]	17/70	24.3[a]	20/64	31.3[a]
0.1	16/62	25.8[ab]	18/66	27.3[a]	21/64	32.8[ab]	24/64	37.5[ab]
0.3	21/63	33.3[b]	18/66	27.3[a]	28/66	42.4[b]	31/64	48.4[b]

Percent mortalities within each column with the same superscript are not significantly different ($p \leq 0.05$).

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the remaining three groups showed a significant increase in mortality rate after exposure to 0.3 mg/m^3 $CuSO_4$ when compared to control mice not exposed to this particulate aerosol. The correlation coefficient for control mice exposed to filtered air was 0.98, for those exposed to NO_2 with superimposed NO_2 peaks 0.99 and those exposed to NO_2 with superimposed NO_2 and O_3 mixture peak the correlation coefficient was 1.00. Thus, a linear relation appeared to be present between the concentration of cupric sulfate aerosol and the mortality due to the streptococcal pneumonia.

Further review of the data also indicated that the most pronounced increase in mortality was observed in mice continuously exposed to NO_2 with the superimposed NO_2 and O_3 peaks. Thus, the stress of this 9 month exposure regimen and the additional insult of a 3 hour exposure to particulate aerosol produced the most severe response. It is also important to note that the effect of cupric sulfate was seen at 0.1 mg/m^3 and 0.3 mg/m^3 . These concentrations of cupric sulfate are markedly below those which, when administered by itself, affected the resistance to streptococcal pneumonia.

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